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Research Article – REVISED VERSION

Insights into the antibacterial mechanism of action of chelating agents by selective deprivation of iron, manganese and zinc

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Running head: Selective metal deprivation by chelating agents

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34 **ABSTRACT**

35 Bacterial growth and proliferation can be restricted by limiting the availability of metal ions
36 in their environment. Humans sequester iron, manganese and zinc to help prevent
37 infection by pathogens, a system termed nutritional immunity. Commercially-used chelants
38 have high binding affinities with a variety of metal ions, which may lead to antibacterial
39 properties that mimic these innate immune processes. However, the modes of action of
40 many of these chelating agents in bacterial growth inhibition and their selectivity in metal
41 deprivation *in cellulose* remain ill-defined. We address this shortcoming by examining the
42 effect of eleven chelators on *Escherichia coli* growth and their impact on the cellular
43 concentration of five metals. Four distinct effects were uncovered: i) no apparent alteration
44 in metal composition, ii) depletion of manganese alongside reductions in iron and zinc
45 levels, iii) reduced zinc levels with a modest reduction in manganese, and iv) reduced iron
46 levels coupled with elevated manganese. These effects do not correlate with the absolute
47 known chelant metal ion affinities in solution, however, for at least five chelators for which
48 key data are available, they can be explained by differences in the relative affinity of
49 chelants for each metal ion. The results reveal significant insights into the mechanism of
50 growth inhibition by chelants, highlighting their potential as antibacterials and as tools to
51 probe how bacteria tolerate selective metal deprivation.

52 **IMPORTANCE**

53 Chelating agents are widely used in industry and consumer goods to control metal
54 availability, with bacterial growth restriction as a secondary benefit for preservation.
55 However, the antibacterial mechanism of action of chelants is largely unknown, particularly
56 with respect to the impact on cellular metal concentrations. The work presented here
57 uncovers distinct metal starvation effects imposed by different chelants on the model
58 Gram-negative bacterium *Escherichia coli*. The chelators were studied both individually
59 and in pairs with the majority producing synergistic effects in combinations that maximise

60 antibacterial hostility. The judicious selection of chelants based on contrasting cellular
61 effects should enable reductions in the quantities of chelant required in numerous
62 commercial products and presents opportunities to replace problematic chemistries with
63 biodegradable alternatives.

64 INTRODUCTION

65 Several transition metals are essential micronutrients for all organisms. An intricate
66 balance of each has to be maintained to avoid deficiency or the toxic consequences of
67 excess. Nutritional immunity, a component of the human innate immune system, makes
68 use of the sequestration of metal ions in order to combat bacterial proliferation by starving
69 such microorganisms of the metal ions they require (1, 2). The bioavailability of iron,
70 manganese and zinc, in particular, is severely constrained in the human body. Bacteria
71 attempt to counteract this host-mediated metal starvation by upregulating metal selective
72 importers and synthesising and exporting their own chelators, such as enterobactin, to
73 assist in metal uptake (2, 3).

74 Synthetic chelating agents form stable complexes with a variety of metal ions and they
75 have the potential to mimic the metal starvation and bacterial growth restriction conditions
76 produced by nutritional immunity. Chelants are widely used in industry, with global
77 consumption of aminopolycarboxylates (e.g. DTPA, EDTA) alone estimated at 200,000
78 tonnes per annum at the beginning of the century (4). Myriad applications include water
79 softening, effluent treatment, paper and textile manufacture, fertilizers, soil remediation,
80 food processing, pharmaceuticals, medical detoxification, cosmetics and detergents,
81 soaps and disinfectants employed in both industrial and domestic settings (5, 6). In many
82 cases, chelants function as potentiators that assist preservation and thus extend the shelf
83 life of products (7-10). Despite their importance in product formulations, the antibacterial
84 mechanism of action of chelating agents has received little attention in recent years, with
85 current knowledge relying on studies concentrating on the consequences for bacterial
86 outer membrane integrity (9). Experiments with the broad-spectrum chelating ligand
87 EDTA (ethylenediamine tetra-acetate) suggest that it damages *E. coli* by disrupting
88 membrane permeability, possibly through the sequestration of Ca(II) and Mg(II) ions that
89 stabilise the lipopolysaccharide (LPS) at the bacterial outer surface (9, 11-14). Treatment

90 of *E. coli* with EDTA enhances susceptibility to various compounds, including amines (15)
91 and antibiotics (16-19), consistent with interference with outer membrane permeability
92 (20, 21). Similar observations have been made with other Gram-negatives, including *P.*
93 *aeruginosa* (17, 22-25). Cell envelope damage by EDTA has been directly visualized by
94 atomic force microscopy of both *E. coli* (26) and *P. aeruginosa* (27). Destabilisation of
95 artificial lipid membranes has also been reported as a consequence of EDTA exposure
96 (28). Few reports have been published on the effect of any other chelants on bacteria,
97 and none appear to have examined their impact on metal homeostasis.

98 In this study, we sought to probe the effects of several different chelating ligands on
99 metal ion acquisition in the archetypal Gram-negative bacterium *E. coli*. Impetus for such
100 a study arose from the use of chelants as bacteriostatic agents in a variety of consumer
101 products and an eagerness to develop alternatives to ligands such as EDTA which largely
102 resist biodegradation (29). To this end, we have characterised the influence of eleven
103 chelants on *E. coli* growth, individually and in combination, and determined their impact
104 on total cellular metal ion concentrations. Our key objectives were to: i) identify the
105 specific metals affected by chelant exposure and their contribution to bacterial growth
106 restriction, ii) assess chelators as potential probes for metal homeostasis that mimic
107 nutritional immunity processes, iii) explore the potential of combinations of chelating
108 agents in antibacterial hostility, and iv) to begin to rationalise such observations in relation
109 to bacterial metallostasis.

110 RESULTS AND DISCUSSION

111 **Chelant selection and inhibitory effects on *E. coli* growth.** Eleven chelators were
112 selected based on their known or predicted metal ion affinities (30-32) and differing
113 chemical structures that might elicit a variety of complementary cellular effects (Fig. 1;
114 Table S1). Most of the chelants are commonly known by their abbreviations rather than
115 their full chemical names. The selection includes EDTA (hexadentate), its octadentate

analogue DTPA, and closely related biodegradable aminocarboxylates GLDA and MGDA, all of which are expected to bind a broad range of metal ions strongly, especially Fe(III). Metal ion affinities are quantified in terms of stability constants (association constants), namely the equilibrium constant K_A for the equilibrium $M + L \rightleftharpoons ML$ at a given pH, ionic strength and temperature, typically expressed as $\log K_A$. Where available, $\log K_A$ values for the selection of chelants used in this study for a number of biologically relevant divalent cations in combination with Fe(III) are listed in Table S1. The metal ion affinities of GLDA and MGDA (29) are lower than those of DTPA and EDTA (Table S1), indicating that higher concentrations may be required to chelate biologically relevant metal ions. DTPMP has a similar nitrogenous backbone to DTPA but possesses five pendant phosphonates – $P(O)(OH)_2$ instead of carboxylates $-C(O)OH$. HBED is another aminocarboxylate, but it also incorporates phenolic units that favour binding to Fe(III) (31, 33). Catechol (CAT; a unit that occurs in enterobactin) has very high selectivity for Fe(III) *in vitro* (34), although its effective binding strength at pH 7 is attenuated due to competitive protonation. CHA is a simple hydroxamate that resembles the constituent binding units of the siderophore desferrioxamine, which binds Fe(III) extraordinarily strongly (35). Piroctone (the metal binding unit of piroctone ethanolamine, PO) is a related cyclic hydroxamate. TPEN and BCS are “softer” ligands that favour binding to late transition metals such as Zn(II) (36) and Cu(I) (37), respectively.

The effect of each of the 11 chelants on bacterial growth was evaluated using 2-fold serial dilutions of each ligand (Fig. 2). The *E. coli* K-12 strain BW25113 was chosen to allow comparisons with deletion mutants from the Keio collection, a comprehensive set of single-gene knockout mutants (38). LB (Lennox) broth was selected as the growth medium, as it is widely used in cultivation of *E. coli* and offers good reproducibility. The provision of a rich growth medium with no inorganic nutrient restrictions (39) allowed an assessment of sensitivity to chelants when bacteria are in a robust physiological state.

Bacterial growth was evaluated based on the optical density at 600 or 650 nm after incubation with the chelant(s) for 16 h as in minimum inhibitory concentration (MIC) determination assays (40). One of the chelants tested, BCS, failed to inhibit bacterial growth fully even at the highest concentrations tested (Fig. 2). The dose-response curves with different chelants also varied, with several chelants (CAT, CHA, GLDA, MGDA and PO) exerting little effect on growth until a particular threshold concentration was reached. Others (DTPA, DTPMP and EDTA) resulted in higher susceptibility at low chelant concentrations and produced a correspondingly linear reduction in growth (Fig. 2). These different sensitivity profiles could be an indication of dual antibacterial effects, such as metal starvation coupled with membrane permeabilization, invoked previously as an explanation for the biphasic inhibition profile of EDTA with *P. aeruginosa* (8). DTPA and EDTA share similar molecular structures (Fig. 1) that may correspond to an analogous mechanism of growth inhibition. In most cases, high concentrations were required to achieve *E. coli* growth inhibition of >90%. To validate these findings, the experiments were repeated with the chemically-defined MOPS-minimal medium supplemented with glucose as the sole carbon source (41). In general, a similar pattern of effects was observed in comparison with the more complex LB broth (Fig. S1). The minor changes seen with BCS, HBED and TPEN may reflect differences in the quantities or relative proportions of the metals present in each medium (see below). The MICs were also similar (Fig. 2), although 6-fold less CAT and 10-fold less EDTA were required to inhibit the growth of *E. coli* in the minimal medium relative to LB. The two chelants with highest efficacy in both media were PO and TPEN with MICs of 75 and 400 μ M in LB and 250 and 200 μ M in MOPS-minimal media, respectively (Fig. 2). PO activity is, however, ambiguous owing to it being comprised of two components. We separated the piroctone from the ethanolamine and found that the former induced growth inhibition comparable to PO, whereas the latter was around 500-fold less active (Fig. S2). Thus, it is the piroctone fragment that is functionally

168 active in bacterial growth reduction, and its activity can reasonably be attributed to its
169 chelating ability.

170 The concentration of metal ions in the LB (Lennox) medium used was determined by
171 inductively-coupled plasma mass spectrometry (ICP-MS; Fig. S3) to provide insight into
172 availabilities prior to examining the effect of chelants on cellular metal content. The metal
173 composition corresponds well with estimates from previous studies using LB (Miller) broth
174 (Fig. S3) (42, 43). The metal content of MOPS-minimal medium was also analysed and
175 found to contain 3.5-fold more magnesium and 2.4-fold more iron, but 19.5 times less
176 calcium than LB (Lennox). Interestingly, the levels of zinc were below the threshold of
177 detection (Fig. S3), although these low concentrations are not likely to be limiting for *E. coli*
178 (44).

179 **Total cellular metal content of *E. coli* exposed to metal chelators.** In order to probe
180 the effect of chelants on cellular metal composition we exposed *E. coli* to concentrations of
181 each ligand that resulted in 10-15% growth inhibition in mid-log phase in LB (Lennox)
182 media. Modest growth inhibition rates were chosen to avoid cellular damage that could
183 potentially skew metal content measurements, owing to increased permeability or cell
184 death. In addition, growth reductions at such low chelant concentrations can be
185 reasonably correlated with cellular metal deprivation. It should be noted that chelating
186 agents that associate with the envelope or reach the cytosol could sequester metals but
187 these cannot be differentiated from the bioavailable metals also present within the cell.
188 Hence, any decreases detected in cellular metal content must be primarily caused by
189 depletion of metals from the extracellular environment or from the bacterial exterior
190 surface. The total number of calcium, iron, magnesium, manganese and zinc ions in each
191 cell was determined using ICP-MS. Copper was also measured but its low concentration
192 made determination less accurate and more prone to variation. Analysis of cobalt and
193 nickel was not undertaken due to the extremely low levels present in *E. coli*. Cobalt is not

194 required by *E. coli* (45) and nickel is only utilised by a small number of [NiFe] hydrogenase
195 isozymes (46). Bacteria were grown in the presence of each chelant, harvested in mid-
196 exponential phase and the total cellular metal composition, expressed in atoms per cell,
197 was determined relative to controls in the absence of the chelant (Fig. 3 and Table S2).

198 Four distinct categories of effect on cellular metal content were identified, primarily
199 through differential effects on zinc, iron and manganese concentrations (Fig. 3), and the
200 results are discussed below according to these functional groupings. It is notable that
201 cellular levels of calcium or magnesium were largely unaffected by exposure to each of the
202 ligands.

203 **(i) No apparent effect on cellular metal content – BCS, CAT and CHA.** The results
204 with BCS, CAT and CHA were unexpected in that they showed no significant impact on
205 the metal composition of *E. coli* cells (Fig. 3; Table S2); these chelants also had no effect
206 on copper levels, although the results with BCS were highly variable (Table S2). Either
207 they act by a completely different mechanism to restrict bacterial growth (i.e. not involving
208 the perturbation of metal availability) or, perhaps more likely, they sequester metals within
209 the cell making them inaccessible to the proteins that require them for functionality. This
210 could potentially occur at the inner or outer membrane, the periplasm or in the cytosol,
211 depending on whether the chelator can traverse the cell wall barrier. It is suggested that
212 chelant-membrane interaction might be more likely in these cases, based on the
213 lipophilicity of these ligands, as reflected by estimations of their partition coefficients in
214 their most likely ionisation states at neutral pH. For example, the long hydrophobic tail and
215 polar head in CHA could potentially insert into the outer membrane and thereby trap
216 essential metals at the surface so they cannot gain access to the cell.

217 **(ii) Decreased manganese, iron and zinc – DTPA, EDTA, GLDA and MGDA.** The
218 principal effect of the azacarboxylate ligands DTPA, EDTA, GLDA and MGDA at 10-15%
219 growth inhibition is to deplete *E. coli* of manganese, with the reductions ranging from 5- to

220 15-fold relative to untreated controls (Fig. 3; Table S2). Zinc concentrations were also
221 reduced at relatively low concentrations of each of these chelants (Fig. 3; Table S2). The
222 total cellular content of iron was also lowered with DTPA, EDTA and GLDA, but not
223 significantly with MGDA (Fig. 3; Table S2). Small reductions in calcium levels were
224 apparent with EDTA and GLDA. The preferential targeting of manganese is surprising
225 given that these chelants would be expected to show a clear preference for iron based on
226 log K_A values (Table S1). There are a number of manganese-dependent enzymes in *E.*
227 *coli* that could be rendered inactive by manganese starvation, including Mn-superoxide
228 dismutase SodA (47), Mn-dependent ribonucleotide reductase NrdEF (48) and the haem
229 biosynthetic enzyme coproporphyrinogen III oxidase HemF (49). Mismetallation of these
230 enzymes (45, 50) and loss of the antioxidant properties of manganese could result in cells
231 being more prone to damage by reactive oxygen species (51). However, low levels of
232 manganese are not problematic for *E. coli* cells unless iron is scarce or they are exposed
233 to hydrogen peroxide (52). Hence, the additional reductions in iron and zinc, alongside
234 manganese depletion, likely impact on multiple metabolic systems and disrupt
235 compensatory pathways for metal import (see below). We investigated this further by
236 supplementing cultures with manganese chloride in the presence of EDTA (Fig. S4). Both
237 EDTA and Mn(II) cause *E. coli* growth inhibition in a concentration-dependent manner (Fig.
238 S4A and B). When EDTA and Mn(II) are mixed at different ratios, improved growth was
239 observed (Fig. S4C and D) consistent with reversal of the cellular manganese deficiency.
240 However, this response could simply be a consequence of EDTA-Mn(II) association in the
241 medium, with the complexes formed moderating the adverse effects associated with EDTA
242 metal sequestration. Supplementation of EDTA-treated *P. aeruginosa* and *S. typhimurium*
243 cells with Ca(II) and Mg(II) has been previously reported (7, 17, 21, 53), with the positive
244 effects attributed to either membrane stabilisation or alleviation of the detrimental EDTA
245 excess by chelant-metal binding.

246 **(iii) Decreased iron and elevated manganese – DTPMP, HBED and PO.** DTPMP,
247 HBED and PO affect cells similarly to one another, reducing cellular iron concentration
248 coupled with a substantial *increase* in manganese (Fig. 3; Table S2). There was no
249 significant change in levels of calcium, magnesium or zinc (Fig. 3). *E. coli* cells are known
250 to import manganese as a cellular response to iron starvation (45, 52). Manganese-
251 equivalents of iron-redox enzymes, e.g. Mn-superoxide dismutase (47, 54) and Mn-
252 dependent ribonucleotide reductase (48), can substitute for iron-containing equivalents,
253 while manganese can functionally substitute for iron in many mononuclear iron enzymes
254 (45, 55). Iron and manganese metal homeostasis systems are linked *via* the ferric uptake
255 regulator (Fur) and the proton-dependent manganese importer MntH (56). The *E. coli* Fur
256 protein, when complexed with Fe(II), represses the expression of a suite of genes involved
257 in iron uptake, metabolism and bacterial virulence (57, 58). Thus, when iron levels are
258 limiting, the affinity of Fur for its promoter sites is reduced leading to upregulation of the
259 iron homeostasis network. One such gene negatively regulated by Fur-Fe(II) is *mntH*, in
260 accordance with the cellular response that switches to manganese import when iron is
261 scarce (56, 59). The manganese superoxide dismutase (MnSOD) is similarly negatively
262 regulated by Fur-Fe(II), whereas Fur-Fe(II) activates expression of iron superoxide
263 dismutase, FeSOD (60, 61). Hence, as iron levels in the cell decrease, FeSOD levels
264 decline just as MnSOD levels rise, concomitant with increased manganese uptake. The
265 decreased levels of iron combined with increased manganese induced by DTPMP, HBED
266 and PO can reasonably be explained by bacterial adaptation to protect against iron
267 starvation.

268 To further investigate the contrasting effects of PO and EDTA on cellular iron and
269 manganese levels, we examined expression of the manganese importer by monitoring β -
270 galactosidase activity from a reporter strain, SIP879, carrying an *mntH-lacZ* fusion (59).
271 Interpretation of the experimental data is complicated by the fact that *mntH* is regulated by

272 both MntR, the manganese regulator, and Fur so we also tested a strain, SIP943, that
273 lacks both *mntH* and *mntR* (59). MntR is a repressor of *mntH* promoter activity under
274 manganese replete conditions (59, 62). Treatment of SIP879 with PO induced expression
275 of the *mntH-lacZ* promoter (Fig. S5A), a typical cellular response to iron starvation (59,
276 62). Similar expression levels between the *mntH-lacZ* and *mntH-lacZ mntR* strains
277 exposed to PO (Fig. S5A) is also consistent with this being a Fur-mediated response to
278 iron deprivation. Hence, iron restriction by PO would be expected to trigger manganese
279 import by MntH, corroborating earlier findings (Fig. 3). The experiments were repeated
280 with EDTA (Fig. S5B) as a representative of chelants that severely restrict cellular
281 manganese concentration, alongside reductions in iron and zinc (Fig. 3). Interestingly,
282 EDTA treatment resulted in activation of *mntH* (Fig. S5B), producing similar effects to PO
283 and indicating that both chelants deprive cells of iron. As with PO, the levels of *mntH*
284 expression were largely unaffected by the absence of *mntR* (Fig. S5B). EDTA has
285 previously been reported to induce expression of *mntH* in both *E. coli* (59) and *Salmonella*
286 (62). Thus, we can conclude that *E. coli* is subjected to iron starvation following exposure
287 to EDTA. However, unlike the situation with PO, the cells are unable to switch to their
288 regular recovery pathway because EDTA has also effectively removed access to
289 manganese.

290 The effect of EDTA on bacterial growth following manganese chloride supplementation
291 (Fig. S4) was revisited in experiments with the *mntH-lacZ* fusion (Fig. S5C). Inclusion of
292 additional manganese to cells growing in LB broth did not induce expression from the
293 *mntH* promoter as expected since MntR-mediated repression is only alleviated by
294 manganese limiting conditions (59, 62). Increased *mntH-lacZ* expression by EDTA was
295 reduced by addition of manganese chloride, especially at equimolar concentrations (Fig.
296 S5C). Similar results were obtained with SIP943, although lower levels of β -galactosidase
297 activity were detected in response to EDTA in all of these experiments (Fig. S5C). While it

298 is difficult to distinguish improvements in chelant tolerance due to either Mn(II) uptake by
299 cells or removal of chelant toxicity by Mn(II) sequestration in the medium (Fig. S4), the
300 absence of activation of *mntH-lacZ* when EDTA and Mn(II) are mixed in equal quantities
301 argues in favour of the latter.

302 **(iv) Decreased zinc and manganese – TPEN.** The predominant effect of TPEN is on
303 zinc concentration, consistent with its known affinity for Zn(II) (36), although as with the
304 other chelants it will bind a range of other metals (Table S1). At higher concentrations of
305 TPEN, manganese levels are also slightly reduced and may contribute to growth inhibition
306 by TPEN (Fig. 3; Table S2). However, at 300 μ M TPEN there is no reduction in Mn(II)
307 whereas Zn(II) is reduced (1.5-fold). These results indicate that even relatively small
308 reductions in cellular zinc levels may adversely affect *E. coli*, in keeping with earlier
309 findings using zinc-depleted media (44). Microarray analysis of *E. coli* exposed to TPEN
310 (63) links chelant exposure with increased expression of genes regulated by the zinc
311 uptake regulator (Zur) (64) but also those controlled by Fur, implying that TPEN may not
312 be entirely selective for zinc. TPEN is often referred to as a membrane-permeable chelator
313 and has been reported to enter *E. coli* cells (36). Preferential removal of zinc from the
314 extracellular environment can account for the reductions in cellular zinc observed here, but
315 it is likely that intracellular zinc is also sequestered by TPEN and contributes to bacterial
316 growth inhibition.

317 **Effect of chelant combinations on *E. coli* growth.** To gain further insight into the
318 impact on bacterial metal restriction, pairs of chelants were tested based on the
319 supposition that those affecting different metal uptake pathways should be synergistic
320 when combined. The checkerboard, or 2-dimensional, assay provides a simple way to
321 evaluate inhibitory interactions between two soluble compounds and has been widely used
322 to compare efficacies of different antibiotics in combination. The microdilution method used
323 for our MIC assays was adapted with consideration of published protocols for the

324 interpretation of checkerboard results (65, 66). The use of checkerboard assays is
325 complicated with chelating agents because in some cases, bacterial growth is never fully
326 inhibited, unlike with many antibiotics. For instance, BCS at a maximal concentration of
327 100 mM only inhibits *E. coli* growth by 70-80% (Fig. 2). A percentage growth of <10% was
328 chosen as a baseline for minimum inhibitory concentrations, which are needed to calculate
329 a fractional inhibitory concentration (FIC) index. For cases like BCS where <10% growth
330 was not achieved, the maximum concentration of chelant provided the MIC and should be
331 taken into account when assessing results obtained with BCS. Representative examples
332 of synergistic, indifferent (or non-interacting) and antagonistic pairings from our studies are
333 illustrated in Figure 4A-C. Overall 55 chelant pairings were tested and FIC indices
334 determined (Fig. 4D), revealing one antagonistic, 26 indifferent and 28 synergistic
335 combinations by selecting the lowest possible combination of each chelant in cumulative
336 calculations (Supplemental Material Data File S1). Considerably fewer synergistic pairings,
337 only 5 (plus 8 mixed synergistic/indifferent outcomes), were obtained using an average
338 FIC method, although that is not surprising as such an approach employs much stricter
339 criteria for assigning synergy (67) (Fig. S6). Synergistic, indifferent and antagonistic
340 pairings are listed according to their effect on metal content to facilitate comparisons
341 between groups (Fig. S7). DTPA yielded the highest number of synergistic pairings, with 9
342 partners (Fig. 4 and S7). BCS produced the lowest number, displaying synergism only with
343 DTPA (Fig. S7), perhaps because of its limited capacity to fully inhibit bacterial growth
344 (Fig. 2 and Supplemental Material Data File S1).

345 **Comparison of checkerboard and metal composition data.** We predicted that
346 chelant categories that cause similar effects on cellular metal levels would produce
347 indifferent outcomes when combined. Conversely, those with dissimilar effects on metal
348 composition might be expected to yield synergistic results. To some extent this proved to
349 be the case, but there were notable exceptions (Fig. 4 and S7). Although the majority of

the synergistic pairs do indeed match complementary categories of metal deprivation, there are 7 examples (DTPA/GLDA, DTPA/MGDA, DTPMP/HBED, DTPMP/PO, EDTA/GLDA, EDTA/MGDA and GLDA/MGDA) where chelants individually induce analogous cellular responses to metals yet produce synergistic effects in combination. There are also multiple examples of chelants from the different metal effect categories defined earlier that show indifference (e.g. DTPMP/EDTA, GLDA/HBED, MGDA/TPEN). The results suggest that there are several different ways that chelants function in depriving cells of metals, even for those that appear to have the same overall effect. Preferential removal of metal either from the media or at the bacterial surface as a function of chelant structure may account for some of these differences. Alternatively, there may be effects produced by chelant-metal association at membranes or in the cytosol that influence metal accessibility. It is interesting to note that CAT and CHA display an identical pattern of synergistic and indifferent outcomes with 7 other chelants and are also indifferent with each other (Fig. S7). These findings suggest that CAT and CHA are functionally equivalent in depriving cells of the same subset of metals despite their dissimilar structures (Fig. 1). This is informative since neither of these chelants appeared to affect total cellular metal content (Fig. 3).

Phenolic compounds, such as CAT, are known to form brown complexes with Fe(III) with absorbance between 380-800 nm (68) and this was apparent when CAT was mixed with media in the presence (Fig. S8A) or absence (Fig. S8B) of bacteria. Different chelant combinations with CAT exacerbated or alleviated the formation of these coloured complexes (Fig. S8A). Those chelants that deprive cells of iron (Fig. 3), such as HBED and PO, appear to reduce the formation of this complex as judged by a loss of colour. In contrast, those predominantly affecting manganese, such as EDTA and GLDA, promote the formation of the dark brown colour (Fig. S8A). The comparatively high concentrations of these chelants, coupled with their relative affinities for different metals, likely serves to

376 remove competing metals from the media, thereby making iron more available for
377 sequestration by CAT. Depending on their commercial application, certain chelant
378 combinations might be best avoided because of the production of pigment, although at
379 lower concentrations this may not be problematic.

380 TPEN is synergistic with all but four chelants (BCS, EDTA, GLDA and MGDA),
381 indicating that reductions in cellular zinc levels might be highly effective as a means of
382 restricting bacterial growth when combined with chelants that primarily limit the availability
383 of other metals. Four chelant pairings (DTPA/DTPMP, DTPA/HBED, DTPA/PO and
384 EDTA/PO) that mainly reduce manganese or iron levels produce synergistic outcomes,
385 although many more chelants from these two categories do not (Fig. 4 and S7). Membrane
386 damage associated with EDTA (9, 14, 20), and potentially with the structurally-related
387 DTPA, may serve to drive partner chelants across the bacterial outer membrane and allow
388 targeting of the periplasm or cytosol. This might account for DTPA/GLDA and EDTA/GLDA
389 synergism, despite all three having similar effects on deprivation of cellular manganese,
390 zinc and iron. In addition, some chelants (e.g. HBED, PO) are somewhat lipophilic and
391 could associate better with membranes, particularly if the LPS layer is damaged. This fits
392 with the iron-binding ligands PO and HBED being synergistic with the hydrophilic DTPMP,
393 another iron chelator (Fig. 4 and S7). Hence the effect of metal starvation coupled with
394 membrane damage or penetration could be instrumental in the bacterial growth restriction
395 phenotype seen with these chelating agents.

396 **Analysis of bacterial metal content with chelants in combination.** To further
397 understand how chelant combinations exert synergistic effects, we selected two
398 synergistic pairs, DTPA/PO and DTPMP/PO, which show distinct effects on cellular metal
399 composition. A fixed concentration of the first chelant producing ~10% bacterial growth
400 inhibition was employed with increasing amounts of the second chelant to produce a 10-
401 30% final growth restriction. As before, the cellular levels of calcium, iron, magnesium,

402 manganese and zinc were determined using ICP-MS (Fig. 5). Selected results showing the
403 proportional change in metal content from experiments with PO in combination with either
404 DTPA or DTPMP are also shown in Table S3.

405 DTPA and PO have a radically different impact on the metal composition of *E. coli* and
406 function as a highly synergistic pair (Fig. 4); DTPA depletes cells of manganese, alongside
407 reductions in iron and zinc, whereas PO increases manganese in response to iron
408 limitation (Fig. 3). We predicted that synergy might be due to DTPA preventing the influx of
409 manganese induced by PO. However, the results showed that the effect of PO seems to
410 dominate over that of DTPA, yielding results similar to PO alone (Fig. 5A; Table S3).
411 Modest increases in calcium were evident at a few concentrations of both chelants (Fig.
412 5A), but there were no significant changes in other metals comparing PO alone with the
413 PO-DTPA combination. As suggested above, the potential influence of DTPA on
414 membrane integrity could exacerbate the activity of the lipophilic PO.

415 In contrast to the DTPA/PO pairing, DTPMP and PO behave similarly in reducing levels
416 of iron and increasing manganese, yet display a synergistic effect on *E. coli* growth where
417 an indifferent response was anticipated. There was little change in metal levels between
418 the effect of DTPMP alone and samples that combined DTPMP with increasing amounts of
419 PO, apart from some reduction in zinc at lower PO concentrations (Fig. 5B; Table S3). It
420 should be noted that a small, but significant, reduction in zinc was evident with DTPMP
421 (Fig. 5B) that was not detected previously with this chelant (Fig. 3). To probe this further,
422 the reciprocal experiment was performed using a fixed concentration of PO and titration of
423 DTPMP (Fig. 5C; Table S3). In this case, a significant reduction in zinc was evident at all
424 concentrations of both chelants relative to PO alone. Although the results are not
425 statistically different due to variability in the data sets, there was also a consistent
426 decrease in iron and increase in manganese when the chelants were combined (Fig. 5C,
427 compare the symbols for each data set). These results are in keeping with DTPMP and

428 PO producing the same effects on cellular levels of manganese and iron, but an additional
429 reduction in zinc when combined. This latter effect may be responsible for the synergism
430 observed between these two chelants (Fig. 4).

431 **Effect of PO on the growth of *E. coli* mutants from the Keio collection.** To provide
432 insight into the gene products important for tolerating exposure to chelants we next
433 selected one of the iron chelators, PO, in a screen of the *E. coli* Keio collection of single
434 gene deletions to identify mutants with increased susceptibility. The duplicate set of the
435 Keio collection of 3985 mutants (7970 strains in total) was cultivated in 96-well plates in LB
436 media in the presence of low levels of PO at 27 and 34 μ M. The growth of each strain
437 exposed to PO relative to untreated controls was determined after overnight incubation
438 and the most sensitive mutants identified (Fig. 6A; Supplemental Material Data File S2).
439 The influence of EDTA on *E. coli* growth has previously been analysed by inoculating the
440 Keio collection mutants onto LB agar plates (69) and this facilitated comparisons with our
441 data on PO (Fig. 6B). The Keio screen with PO highlighted the importance of genes
442 involved in iron-siderophore uptake for PO tolerance (Fig. 6C). Mutants affecting
443 enterobactin synthesis (Aro, Ent), export (TolC) and import (FepA-G, ExbBD-TonB and
444 Fes) were among those with the most substantial growth reductions relative to the control
445 following PO exposure (Fig. 6A and 6C). Deletion mutants affecting envelope integrity,
446 efflux pumps, damage tolerance and stress responses also showed sensitivities to PO
447 (Fig. 6A), potentially indicating that PO can more readily gain access to the periplasm or
448 cytosol in these strains and thereby affect growth. Some similarities in growth behaviour
449 with EDTA (69) were observed with a similar subset of genes involved in enterobactin-iron
450 uptake and membrane integrity affected. However, unlike PO, mutants defective in
451 components of the Znu zinc uptake system showed impaired growth when exposed to
452 EDTA (Fig. 6B).

453 A small number of mutants displayed improved growth relative to untreated controls
454 when PO was incorporated in the growth medium (Table S4). Several of these mutants
455 display better growth at both PO concentrations suggesting that their deletion does
456 correspond to a genuine improvement in growth. These mutants correspond to genes
457 linked to regulatory pathways, metabolic processes and repair of oxidative damage.
458 However, the largest group of mutants affected are those engaged in flagellar
459 biosynthesis, of which 26 *fli*, *flg* and *flh* genes occur in the 200 mutants that show the most
460 enhanced growth at both PO concentrations (Table S4). This may represent an alleviation
461 of the substantial energy cost involved in flagellum assembly and operation (70) during the
462 iron limitation imposed by PO. Significantly, flagellar gene-deficient mutants do not exhibit
463 the most enhanced growth of the Keio mutant strains under low iron conditions using
464 MOPS media (69) suggesting that PO either exerts additional detrimental effects or targets
465 iron depletion with a different cellular specificity.

466 **Effect of PO, EDTA and DTPMP on the growth of selected *E. coli* Keio collection**
467 **mutants.** To validate the findings with the Keio screen, we selected a range of the most
468 PO susceptible mutants and others deficient in related iron, manganese and zinc uptake
469 pathways for further testing. All of the mutants affecting enterobactin biosynthesis or
470 uptake (*aroA*, *fepA*, *fepC* and *fes*) (71) exhibited substantially reduced growth relative to
471 the wild-type following exposure to PO (Fig. 7A), consistent with the importance of iron
472 acquisition for tolerance of this chelant. Interestingly, a corresponding sensitivity was not
473 found with *fepB* and *fepD* mutants (Fig. S9A). Several strains lacking integral membrane
474 proteins involved in drug export and envelope integrity (*acrB*, *envC* and *tolC*) also showed
475 some increased susceptibility as in the Keio screen with PO (Fig. 7A). Two mutants, *znuB*
476 and *znuC*, affecting zinc import (72) behaved similarly to the wt as expected. Mutants
477 affecting components of the Fe(III)-citrate (*fecA*, *fecB*, *fecD* and *fecE*) and Fe(III)-
478 hydroxamate (*fhuF*) systems (73) were generally no more susceptible to PO. Similarly,

mutants involved in cysteine (*cysE*) and histidine (*hisI*) biosynthesis that are highly sensitive to iron starvation (69), showed no increased susceptibility to PO (Fig. S9A).

The same strains were also examined for their susceptibility to DTPMP and EDTA (Fig. 7B and 7C), the latter allowing comparisons with published data (69) that were conducted on solid rather than liquid media. As with PO, defects in the enterobactin pathway (*aroA*, *fepA*, *fepC* and *fes*) produced the highest sensitivity to these two chelants, underlining the necessity of this route of iron acquisition for bacterial growth and defence against these chelants. In contrast to PO, the other ferric iron import pathway mutants also showed increased susceptibility, especially with DTPMP (Fig. S9B and S9C). Reduced growth following chelant exposure was apparent with mutants affecting membrane integrity functions.

Mutants in the *znuB* and *znuC* zinc import system were much more sensitive to DTPMP and EDTA (Fig. 7B) than PO (Fig. 7A), suggesting that reductions in cellular zinc – either due to mutation or sequestration by a ligand such as TPEN – increase chelant vulnerability. DTPMP treated cells display low levels of iron and elevated concentrations of manganese (Fig. 3), however, small reductions in zinc were also apparent, especially when combined with PO (Fig. 5B). The enhanced susceptibility of *znu* mutants to DTPMP but not PO indicates that these two chelants do not behave precisely in the same way and that additional effects on zinc may account for their synergistic behaviour (Fig. 4). Deletion of the manganese importer, *MntH*, did not result in decreased growth following EDTA exposure (Fig. S9C) in agreement with previous studies (69). In repeat assays, growth was actually improved following EDTA treatment in an *mntH* strain (Fig. S10). Although it is not clear why growth would be improved in the absence of *mntH*, these observations are consistent with combined reductions in iron, manganese and zinc, rather than manganese alone, being important for bacterial growth inhibition by EDTA.

CONCLUSIONS

505 Using *E. coli* as a model organism, the specific metals affected by a selection of chelating
506 agents have been identified and their impact on bacterial growth and metal deprivation
507 evaluated. The cellular concentrations of calcium, iron, magnesium, manganese and zinc
508 were determined for eleven chelators with differing structures and metal ion selectivities.
509 Four categories of chelants with distinct effects on metal depletion were identified.

510 BCS, CHA and CAT do not appear to alter the levels of any of the metals tested,
511 although it is possible that they trap particular metals, potentially at the cell surface, and
512 thus prevent metals from accessing the cell. Hence the metals would remain associated
513 with the cell but would be unavailable to essential intracellular enzymes. Of these three,
514 CHA and CAT appear to be functionally equivalent as judged by their similar behaviour in
515 combination with other chelants.

516 DTPA, EDTA, GLDA and MGDA all produce a dramatic decrease in cellular
517 manganese, combined with lesser reductions in both iron and zinc. Iron and zinc limitation
518 could well be the principal factor in bacterial growth inhibition with these chelants since *E.*
519 *coli* mutants with defects in uptake pathways for these metals (e.g. *fepA*, *fes*, *znuB*) are
520 more sensitive to EDTA (Fig. 6 and 7) (69). That manganese has only a secondary effect,
521 perhaps at the cell surface, fits with the improved growth of an EDTA-treated *mntH* mutant
522 (Fig. S10), which lacks the MntH manganese transporter that would boost cytosolic levels
523 of Mn(II) (55). Examination of *mntH* promoter activity (Fig. S5) confirmed that EDTA
524 starves cells of iron, but is also likely to prevent manganese import by sequestration
525 making this route of tolerance ineffective. EDTA, and potentially DTPA, has known
526 detrimental effects on outer membrane integrity (9, 27), meaning that a combination of
527 metal starvation and membrane damage likely contributes to its antibacterial mechanism
528 of action. It is feasible that stripping of manganese from a primary location at the bacterial
529 surface is responsible for the injurious effects on membrane stability. The cellular location

530 of manganese has yet to be established (55), although in the Gram-positive *Bacillus*
531 *subtilis*, Mn(II) does appear to be associated with the cell wall (74).

532 Exposure of *E. coli* to DTPMP, HBED and PO causes a reduction in iron and an influx
533 of manganese; the triggering of manganese import is a known cellular defence response
534 to iron starvation (45, 52) in keeping with these ligands being Fe(III) chelators.
535 Experiments with combinations of these three chelants, however, suggest that they are not
536 functionally equivalent and that their cellular targets may differ. Cells deficient in the zinc
537 (Znu) uptake and ferric-citrate (Fec) pathways are hypersensitive to DTPMP but not PO
538 (Fig. 7 and S9) and reductions in cellular zinc levels were apparent with DTPMP,
539 especially when mixed with PO (Fig. 5). As with DTPA, EDTA, GLDA and MGDA, the
540 potential for membrane penetration or damage may account for the differing interactions
541 observed.

542 Why might certain chelants, such as EDTA and DTPA, deplete cells of manganese
543 considerably more than iron? Affinities for both Fe(III) and Mn(II) are known for five of the
544 chelants which interfere with the accumulation of these metals, namely DTPA, EDTA,
545 GLDA, HBED and MGDA (Table S1). Figure 8 shows their relative affinities (as
546 $\log(K_{\text{Fe(III)}}:K_{\text{Mn(II)}})$ where K_{metal} correspond to association constants K_A) along with
547 comparative estimated values for uptake systems for these two metals: note that an
548 available $K_{\text{Mn(II)}}$ from *S. aureus* MntC has been used in the absence of a measured value
549 for *E. coli* MntH, and a pseudo- K_A for Fe(III)-citrate₂ was simulated for defined total Fe(III)
550 and citrate concentrations (1 μM and 100 μM , respectively; (75)). Importantly, only
551 $\log(K_{\text{Fe(III)}}:K_{\text{Mn(II)}})$ for HBED exceeds estimated values for all uptake systems (Fig. 8), and
552 of the five chelants only HBED impairs the uptake of iron and not manganese (Fig. 3).
553 Thus, even if two chelants show tightest affinity for the same metal, their relative affinities
554 (for different metals) can drastically alter their impact on cellular metal acquisition systems.
555 This preliminary analysis suggests that it may be possible to model bacterial responses to

556 chelants based on relative metal affinities and by measuring K_A for all uptake systems for
557 all metals to predict cellular responses to chelants.

558 As outlined above, analysis of the cellular metal selectivity of the chelants tested
559 allowed the identification of chelants with high specificity for iron, manganese and zinc that
560 could serve as mimics of nutritional immunity and as tools to probe bacterial metal
561 homeostasis. Those with specificity for zinc and iron offer clear value although those
562 affecting manganese may exhibit too broad a range of metal target. There is considerable
563 potential to exploit two, or even three, chelants to restrict bacterial growth in a range of
564 consumer, industrial and healthcare settings. For example, CHA, EDTA and PO acting by
565 different mechanisms could prove a potent antibacterial mixture. Chelators could be
566 deployed in combination with antibiotics for wound care and other therapeutic applications,
567 especially as they are implicated in disrupting biofilm formation (27, 76, 77). Metal toxicity
568 could also be harnessed in the presence of chelants that selectively restrict availability of
569 iron, manganese and zinc to mimic the killing achieved in phagocytic vesicles (78).
570 Modelling bacterial uptake of metals will assist in identifying the specificity of molecules for
571 manipulating metal acquisition. Affinity ratios can identify which chelants preferentially
572 interfere with which metals for uptake as exemplified by $\log(K_{Fe(III)}:K_{Mn(II)})$ values for the
573 iron-selective chelator HBED (24.9) which exceeds values estimated for Fe(III) versus
574 Mn(II) uptake (22.1), and exceeds values for DTPA, GLDA, EDTA and MGDA (≤ 14.4)
575 which preferentially target manganese.

576 The results from this study show that in most cases it is challenging to predict,
577 especially from available empirical metal ion affinity data, which combinations are likely to
578 be most effective (79). However, we now have a much clearer understanding of the metals
579 affected and indications that the cellular sites of metal sequestration may differ between
580 them. Significantly, a large number of synergistic antibacterial chelant combinations have
581 been identified that could be incorporated into products where their preservation properties

are desirable. New formulations can be manufactured that reduce the quantities of chelants required and integrate biodegradable alternatives (e.g. GLDA/MGDA) with major benefits for sustainability and environmental compatibility. Further work is needed to rationalize our predictive capabilities with chelating agents and define precisely: (i) the localization of chelants within cells, (ii) robust metal ion affinities for chelators to multiple metal ions *in vitro*, (iii) how these affinities compare with the availabilities (buffered concentrations / free energies) of the elements at their respective locations (80), and (iv) whether bacterial species with different cell wall structures and metal uptake strategies exhibit similar cellular responses.

MATERIALS AND METHODS

Bacterial growth inhibition by chelants. Chelating agents were obtained commercially and are listed in Table S5. Most chelants were soluble in water, but CHA, HBED and PO were resuspended in dimethyl sulfoxide (DMSO) and TPEN in ethanol. Appropriate vehicle controls were performed in parallel for all growth experiments involving these chelants. *E. coli* K-12 BW25113 (*rrnB3* Δ *lacZ4787* *hsdR514* Δ (*araBAD*)567 Δ (*rhaBAD*)568 *rph-1*) and deletion-insertion derivatives from the Keio collection (38) were used in this study. For microdilution MIC assays, *E. coli* cultures were grown in LB media (Lennox, Sigma Aldrich) or MOPS-minimal media (Teknova Inc) in an orbital shaker (Stuart) at 37°C to an OD_{650nm} of 0.07, equivalent to a 0.5 MacFarland standard (240 μ M BaCl₂ in 0.18 M H₂SO₄ aq.) and diluted 10-fold in LB broth for use as an inoculum (65). The diluted culture (50 μ l, 5 x 10⁶ CFU/ml) was then transferred into a 96-well, round-bottomed microtitre plate (Sarstedt). Chelants from stock samples, prepared in water, DMSO or ethanol, were diluted to yield a 2-fold series in LB broth and 50 μ l mixed with the diluted inoculum. Plates were incubated at 37°C with shaking at 130 rpm for 16 h and absorbance (A_{600nm} or A_{650nm}) monitored on a Spectrostar Nano plate reader. MICs were defined as the minimum concentration of chelant needed to inhibit growth by >90% relative to controls.

608 Checkerboard assays were performed to assess the effect of chelants in combination.
609 Stock solutions and inoculum were prepared as for MIC experiments. One chelator was
610 applied in decreasing concentrations horizontally across the 96-well microtitre plate, while
611 the second chelator was added in decreasing concentrations vertically to create the
612 checkerboard (Supplemental Material Data File S1). A Fractional Inhibitory Concentration
613 index (FICI) was defined as the minimum concentration of chelant needed to inhibit growth
614 by >90% individually and in combination and FIC index values were interpreted as
615 synergistic (≤ 0.5), indifferent ($> 0.5-4.0$) or antagonistic (> 4) based on published methods
616 (66, 67) and according to the formula shown in Figure 4.

617 **Isolation of piroctone from piroctone olamine.** PO was dissolved in the minimum
618 amount of methanol prior to the addition of 1M HCl (until pH 1 was reached). The mixture
619 was then transferred to a separating funnel, diluted with either DCM or chloroform and the
620 organic layer collected, dried over MgSO_4 and the solvent removed *in vacuo*. Drying the
621 resulting solid to constant weight using a high vacuum line afforded piroctone as an off-
622 white powder. ^1H NMR (400 MHz, DMSO) δ 6.19 (d, 1H), 5.93 (d, 1H), 2.59 (dd, 1H), 2.38
623 (dd, 1H), 2.10 (s, 3H), 2.08 (d, 3H), 2.03 – 1.91 (m, 1H), 1.26 (dd, 1H), 1.08 (dd, 1H), 0.88
624 (d, 3H), 0.82 (s, 9H).

625 **β -galactosidase assays to monitor *mntH-lacZ* promoter activity.** SIP879 (*mntH::*
626 *Mud1(Ap, lac) aroB*) and SIP943 (*mntH::Mud1(Ap, lac) aroB mntR*) are *E. coli* K-12
627 derivatives of MC4100 (*araD139 $\Delta(lacIZYA-argF)$ U169 rpsL150 relA1 flhD5301 deoC1*
628 *fruA25 rbsR22*) (59) and were kindly provided by Laura Runyen-Janecky. Promoter activity
629 assays were performed as described previously (81). Briefly, bacteria were cultivated in LB
630 broth in sterile cuvettes (1 ml) in the presence or absence of chelant or MnCl_2 to an $A_{600\text{nm}}$
631 of 0.5. 80 μl of culture was transferred to a 96-well microtitre plate followed by addition of
632 120 μl master mix (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgSO_4 , 36 mM
633 β -mercaptoethanol, 166 $\mu\text{l/ml}$ T7 lysozyme, 1.1 mg/ml ONPG and 6.7% PopCulture

634 Reagent obtained from Merck Millipore). This was then transferred to a SPECTROstar
635 Nano absorbance plate reader (BMG LABTECH) set to 30°C with shaking at 400 rpm, with
636 absorbance readings taken at 420 and 550 nm every minute for 1 hour. Miller Units were
637 calculated using the following equation $1000 \times [(A_{420\text{nm}} - 1.75 \times OD_{550\text{nm}})] / (T \times V \times A_{600\text{nm}})$
638 where T = time in minutes, and V = volume in ml (0.2).

639 **Keio collection screen.** The duplicate set of 3985 Keio library mutants, 7970 strains in
640 total (38), were grown in 200 µl LB media without antibiotic supplementation at 37°C with
641 27 or 34 µM PO in 96-well microtitre plates for 16 h. A Versette Automated Liquid Handler
642 (ThermoFisher) was used to dispense media and treatments, and inoculate the library.
643 Percentage growth was determined by comparison of $A_{600\text{nm}}$, using a SpectraMax plate
644 reader (Molecular Devices), with untreated controls for each strain.

645 **Determination of cellular metal content.** Different concentrations of chelant were added
646 to 50 ml LB broth in 250 ml acid washed conical flasks prior to inoculation with 1×10^7 *E.*
647 *coli* BW25113 cells. Cultures were grown at 37°C in an orbital shaker at 130 rpm with the
648 aim of inhibiting growth by 10-15% during mid-log phase (~ 0.3 - 0.4 $A_{650\text{nm}}$, typically 3-4
649 hours of growth). Cell numbers were recorded using a Casy Model TT Cell Counter prior to
650 harvesting. Cells were pelleted by centrifugation (19,000 g, 25 min) and resuspended in 50
651 ml wash buffer (0.5 M sorbitol, 10 mM HEPES pH7.8) and centrifuged once again at
652 19000 g for 25 min. The cell pellet was then digested in 5 ml, 65% nitric acid (Suprapur®,
653 Sigma Aldrich) for a minimum of 16 h. These pellet digests were diluted with 2% nitric acid
654 and 5.89×10^{-4} µM silver standard for ICP (Sigma Aldrich) in a 1:8:1 ratio. Calibration
655 samples were made using known quantities of metals in nitric acid (ICP multi-element
656 standards, CertiPUR®, Sigma Aldrich & Merck) diluted in matrix-matched solution.
657 Dilutions and a calibration curve were analysed using inductively coupled plasma mass
658 spectrometry (ICP-MS, Thermo XSERIES 2). Instrument control, analysis and
659 quantification was obtained using software interface PlasmaLab (Thermo Scientific) and

660 further analysis was conducted in Microsoft Excel. Mean and standard deviation values
661 were determined from triplicate biological analyses.

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680

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- 906

907 **Figure legends**

908 **FIG 1** Structure of chelants selected for analysis. BCS (bathocuproine disulphonic acid),
909 CAT (catechol), CHA (caprylhydroxamic acid), DTPA (diethylenetriaminepentaacetic acid),
910 DTPMP (diethylenetriaminepentamethylene phosphonic acid), EDTA
911 (ethylenediaminetetraacetic acid), GLDA (glutamic acid-N,N-diacetic acid), HBED (N,N-bis
912 (2-hydroxybenzyl) ethylenediamine-N,N-diacetic acid), MGDA (methylglycinediacetic acid),
913 PO (piroctone olamine) and TPEN (N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine).
914 The most biodegradable isomer of GLDA (L-GLDA) is shown.

915 **FIG 2** Effect of chelants on *E. coli* growth in LB. Bacteria were cultivated in LB (Lennox)
916 media and mixed with appropriate 2-fold dilutions of each chelant and incubated with
917 shaking for 16 h at 37°C. Results are the mean and standard deviation of an independent
918 experiment performed in triplicate; a further two independent experiments performed in
919 triplicate yielded similar results. MICs in mM are based on >90% growth inhibition, where
920 achieved, as indicated at the bottom right of the figure; they were determined for LB and
921 MOPS-minimal media from 3 and 4 biological replicates, respectively.

922 **FIG 3** Effect of chelants on the metal composition of *E. coli*. Chelants are grouped
923 according to the similarity in their effects on cellular metal concentration (i-iv) as described
924 in the text. Selected results correspond to growth inhibition of 10-15% and the amount of
925 each metal determined in atoms per cell using ICP-MS. BW25113 cells were grown in 50
926 ml of LB pH7 to log-phase in a shaking incubator (125 rpm) at 37°C. Data are the mean
927 and standard deviation of 3 independent biological replicates (one-way ANOVA comparing
928 each chelant concentration with the untreated control in each case, ** $P < 0.01$, *** $P <$
929 0.001 , **** $P < 0.0001$). Concentrations of each chelant are indicated below each set of
930 graphs. The original data were determined over a range of chelant concentrations in most
931 cases and a full summary is provided in Table S2.

932 **FIG 4** Chelant combinations analysed by the checkerboard assay. Examples of (A)
933 synergistic, (B) indifferent and (C) antagonistic pairings, for CHA/DTPMP, CAT/PO and
934 BCS/TPEN, respectively, are shown. (D) FIC index values are shown for two independent
935 experiments performed in triplicate for each chelant combination. The assay allows the
936 calculation of an MIC for each chelant and hence the FIC, which provides a measure of
937 the effect of the chelants in combination as synergistic, indifferent or antagonistic. FIC
938 index values were calculated based on the lowest concentration of each chelant in
939 combination divided by the MIC for that chelant according to the formula shown. Two-fold
940 dilutions (as in an MIC) of chelants were performed in LB broth with *E. coli* BW25113 at
941 37°C with shaking at 37°C for 16 h. Additional controls for low levels of DMSO, ethanol or
942 water were included where relevant.

943 **FIG 5** Effect of selected chelant combinations on total cellular metal content. (A) 14 µM PO
944 with addition of 2, 4, 6, 7, 7.5, 8, 8.5, 9.5 and 10 µM DTPA. (B) 10 µM DTPMP with
945 addition of 12, 13, 14, 15 and 16 µM PO. (C) 15 µM PO with addition of 1.25, 2.5, 3.75, 5,
946 6.25, 7.5, 8.75 and 10 µM DTPMP. Concentrations of each chelant used are indicated
947 below each set of graphs. Results correspond to growth inhibition of 10-30% (grey bars in
948 the topmost graphs) and the amount of each metal determined in atoms per cell using
949 ICP-MS. BW25113 cells were grown in 50 ml of LB to log-phase in a shaking incubator at
950 37°C. Data are the mean and standard deviation of 3 independent biological replicates
951 (one-way ANOVA comparing each chelant concentration with the untreated control in each
952 case or between single chelant treatment with addition of a second chelant as indicated,
953 $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). Although some experiments were subject to
954 variability, there were consistent trends with Fe, Mn and Zn levels in each of the three
955 independent experiments in some cases; for these metal ions, the data points are
956 highlighted as differently-coloured symbols to show the pattern of each of the three
957 replicates.

958 **FIG 6** Effect of PO on the growth of *E. coli* mutants from the Keio collection. (A) The
959 duplicate set of 3985 Keio library mutants (38), 7970 strains in total, were grown in LB
960 media at 37°C with 27 or 34 μ M PO for 16 h. Percentage growth was compared to
961 untreated controls for each strain and the top 50 slowest growing mutants are shown (see
962 Supplemental Material Data File S2). Where two percentages under each condition are
963 shown, these correspond to the presence of both duplicates from the Keio collection in the
964 top 200 slowest growing mutants identified in the screen. Each mutant is colour-coded
965 based on the functional grouping assigned for each gene with the key shown in (C). (B)
966 The 50 slowest growing mutants identified from the Keio phenotypic screen using EDTA
967 (69) is shown to facilitate comparisons with PO. The more negative pixel score values
968 correspond to the poorest colony growth on agar plates supplemented with 0.5 mM EDTA.
969 (C) The ferric enterobactin synthesis, export and import system of *E. coli*. Key proteins
970 involved in each part of the iron uptake system are colour-coded according to their roles
971 (71, 73). AroA-M proteins are involved in the biosynthesis of chorismate that is converted
972 by EntABC to 2,3-dihydroxybenzoic acid (DHB). EntDEF catalyse DHB and L-serine
973 linkage and ultimate assembly into enterobactin (71), which is exported to the extracellular
974 environment by EntS and TolC (82). The ferric-enterobactin complex is recovered by
975 association with the outer membrane receptor FepA. The TonB/ExbBD complex provides
976 energy from the proton motive force to mediate release of the Fe(III)-enterobactin complex
977 from FepA, facilitated by FepB, and delivery to the FepCDG ABC-family, ATP-dependent
978 inner membrane permease (71). Upon reaching the cytosol, Fe(III) is released from the
979 siderophore by the Fes esterase (83). Another ABC-family transporter, ZnuABC,
980 transports Zn(II) across the inner membrane (72, 84). Outer and inner membranes are
981 depicted as lipid bilayers with the lower portion of the diagram shaded in blue to represent
982 the cytosol. Where substantially-reduced growth is associated with mutation of key ferric-

983 siderophore synthesis and transport components, these are indicated with cyan and blue
984 (PO) and green (EDTA) symbols.

985 **FIG 7** Selected *E. coli* mutant sensitivity to (A) PO, (B) DTPMP and (C) EDTA. Bacteria
986 were incubated with a two-fold serial dilution of each chelant in 100 μ l of LB media and
987 incubated with shaking for 16 h at 37°C. Absorbance at 600 nm was recorded and the
988 percentage growth calculated for each strain. Data represent the mean and standard
989 deviation of an independent experiment performed in triplicate. A second biological repeat
990 was performed and a similar pattern of susceptibility was observed.

991 **FIG 8** Relative Fe(III) and Mn(II) affinities of chelants which primarily restrict either Fe(III)
992 (in red) or Mn(II) (in blue) accumulation in cells (judged by % reduction of metal content in
993 chelant-treated cells compared to untreated controls). The relative metal affinities of
994 selected components of uptake systems for iron or manganese at the cell surface are
995 shown by the red dotted lines: The association constants of Fe(III)-enterobactin and
996 Fe(III)-citrate at pH 7.0 were calculated using reported pH-independent affinities of ligands
997 and pK_a values of coordinating atoms (85-87); the Mn(II) affinity of *S. aureus* MntC (solute
998 binding protein) was used in the absence of a known affinity of the *E. coli* manganese
999 transporter MntH (88); a 'pseudo-affinity' of the Fe(III)(citrate)₂ complex was derived from
1000 the calculated pFe^{3+} at pH 7.4 when $[Fe(III)]_{tot} = 1 \mu M$ and $[citrate]_{tot} = 100 \mu M$ (75).















